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Molecular Procedure for Rapid Detection of *Burkholderia mallei* and *Burkholderia pseudomallei*

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A PCR procedure for the discrimination of *Burkholderia mallei* and *Burkholderia pseudomallei* was developed. It is based on the nucleotide difference T 2143 C (T versus C at position 2143) between *B. mallei* and *B. pseudomallei* detected in the 23S rDNA sequences. In comparison with conventional methods the procedure allows more rapid identification at reduced risk for infection of laboratory personnel.

Both *Burkholderia mallei* and *Burkholderia pseudomallei* cause severe infectious diseases in humans, namely, glanders or melioidosis. *B. pseudomallei* is found in soil and water (e.g., rice paddies). Humans can be infected by soil contamination of skin abrasions, ingestion, or inhalation (5). Melioidosis is endemic in Southeast Asia and northern Australia (14). Cases in humans or animals occur sporadically throughout the world. The mortality of untreated infections is high (95% [18]). Glanders is primarily an infectious disease of the horse, mule, or donkey. Glanders in humans is acquired from infected animals or by contact with organisms causing human glanders via ingestion or inhalation (7). Laboratory workers are at high risk to be infected with glanders by aerosols (3). The outcome of untreated infections (e.g., septicemia) is uniformly fatal (7, 18).

The detection and identification of *B. mallei* and *B. pseudomallei* entail a particular risk of infection for laboratory personnel. We established a PCR procedure which allows rapid, less dangerous, and specific identification and discrimination of both species.

Organisms. The strains used are specified in Table 1. Only nonviable material of *B. mallei* and *B. pseudomallei* was available.

Nucleic acid purification. Genomic DNA was purified by using the QiaAmp purification kit (Qiagen, Hilden, Germany).

PCR. Custom oligonucleotide primers were purchased from MWG Biotech, Ebersberg, Germany (Table 2). Amplification reactions were performed in a 50-μl final volume with 1 U of *Taq* polymerase (Boehringer, Mannheim, Germany), 5 μl of the reaction buffer supplied by the manufacturer (diluted 1:10), a 10 μM concentration of each deoxynucleotide triphosphate, and a 50 pM concentration of each oligonucleotide primer. To avoid reading mistakes, the Expand High Fidelity PCR system (Boehringer) with a proofreading polymerase was used.

To enhance the specificity of *B. mallei* identification, a double concentration (100 pM) of a competitive oligonucleotide probe, which covers the respective 23S ribosomal DNA (rDNA) primer binding sites of all *Burkholderia* spp. except those of *B. mallei*, was used. Due to a modification at its 3′ end with an amino linker (MWG Biotech) no PCR products can be amplified with this probe.

Approximately 50 to 100 ng of DNA template was used in each amplification. The PCR was performed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) with an initial denaturation step of 5 min at 95°C followed by 25 amplification cycles of 30 s at 95°C, 30 s at the primer-specific annealing temperature (Table 2) and 45 s at 72°C. The samples were then incubated at 72°C for another 7 min and cooled to 4°C. Double-distilled, sterile water instead of template DNA was used as the negative control to exclude amplicon contamination.

The amplification products were checked by agarose gel electrophoresis and subsequently purified by using the PCR purification kit (Qiagen) to desalt and remove excess primers.

Agarose gel electrophoresis. Aliquots of PCR products were diluted 10:1 in serving buffer (20% Ficoll, 50 mM EDTA) and electrofocused in a 1% agarose gel (BIOzym, Oldendorf, Germany) on a horizontal electrophoresis apparatus (Gibco BRL, Eggenstein, Germany) at 100 V and 150 mA. Gels were stained with ethidium bromide as described by Sambrook et al. (17) and documented digitally with EASY Image Plus, version 4.13 (Herolab, Wiesloch, Germany). Lengths of the PCR products were compared with those of internal PCR product standards and DNA molecular weight markers (Boehringer).

Sequence determination. Sequence analyses of the in-vitro-amplified rDNA genes were performed as described previously (10) with gene-specific primers (10, 16). The two strands of the DNA were sequenced from different PCR products. Sequencing was performed by the dideoxy chain termination procedure using an automatic sequencer (373A; Applied Biosystems, Weiterstadt, Germany).

Analysis of the sequence data. The nucleotide sequences were aligned with reference rDNA sequences provided in the noncommercial program package ARB (beta-version 2.4; e-mail address: arb@mikro.biologie.tu-muenchen.de). The secondary structure analysis was performed as described by Ludwig et al. (11).

16S and 23S rDNA sequences. The nucleotide sequences of the 16S rDNAs of *B. mallei* and *B. pseudomallei* (data from the literature [6, 20, 24] and our own sequence data) were found to be completely identical. So there is no possibility to differentiate *B. mallei* from *B. pseudomallei* at the 16S rDNA level.

So the 23S rDNA was analyzed for sequence deviations

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TABLE 1. *Burkholderia* and *Ralstonia* strains used and evaluation of the specificity of the PCR procedure

Species[a]	Strain or origin[b]	Results of PCR[c] with primers:	
		VPM 23-1 + MP 23-2	CVMP 23-1 + M23-2
B. mallei	ATCC 23344^T	+	+
B. mallei	ATCC 15310	+	+
B. mallei	ATCC 10399	+	+
B. pseudomallei	ATCC 23343^T	+	−
B. pseudomallei	ATCC 15682	+	−
B. pseudomallei	NCTC 1691	+	−
B. pseudomallei	12 clinical isolates[d]	+	−
B. cepacia	LMG 1222^T	−	−
B. cepacia	DSM 50181	−	−
B. cepacia	4 clinical isolates	−	−
B. vietnamiensis	LMG 10929^T	−	−
B. vietnamiensis	LMG 6998	−	−
B. vietnamiensis	LMG 6999	−	−
B. vietnamiensis	Clinical isolate	−	−
B. gladioli	DSM 4285	−	−
B. gladioli	4 clinical isolates	−	−
R. pickettii	ATCC 27511^T	−	−
R. pickettii	Clinical isolate	−	−
R. eutropha	ATCC 17697^T	−	−

[a] *B. gladioli*, *Burkholderia gladioli*; *R. pickettii*, *Ralstonia pickettii*; *R. eutropha*, *Ralstonia eutropha*.
[b] ATCC, American Type Culture Collection, Manassas, Va.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, Laboratorium v. Microbiologie Universiteit Gent, Ghen, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England.
[c] +, 1,051-bp product produced; −, no product produced.
[d] Including *B. pseudomallei* 2 and *B. pseudomallei* B.

appropriate to discriminate between the two species. As no sequence data were available at the time, the complete 23S rDNA gene sequences (2,882 bp) of two *B. mallei* strains (ATCC 23344^T and ATCC 15310) and three *B. pseudomallei* type culture strains were determined (Table 1). The 23S rDNA gene sequences of the two *B. mallei* strains were completely identical (Fig. 1). This was in contrast to the 23S rDNA gene sequences of the *B. pseudomallei* strains, which turned out to be heterogeneous. Nucleotide substitutions in comparison with the *B. mallei* sequence were identified: for *B. pseudomallei* 2, C 541 G (C versus G at position 541), T 542 C, T 543 A, C 544 A, T 1521 G, C 1522 A, C 1526 A, G 1529 T, and A 1530 C; for the *B. pseudomallei* type strain, T 1521 G, C 1523 T, T 1524 C, C 1526 A, and A 1530 C; for *B. pseudomallei* B, no difference except C 2143 T. These substitutions were, however, inadequate for species-specific primers, as they are variable among different strains of *B. pseudomallei*.

A comparison of the 23S rDNA nucleotide sequences of *B. mallei*, *B. pseudomallei*, and other *Burkholderia* species demonstrated that all three *B. mallei* strains carry a thymidine (T) at position 2143 of the 23S rDNA (Fig. 2) in contrast to a cytosine (C) in all strains of the other *Burkholderia* species investigated. So this substitution, C 2143 T, appears unique for *B. mallei* within the *Burkholderia*/*Ralstonia* sublineage (Table 1). This finding provides a possible means for the molecular discrimination of *B. mallei* from *B. pseudomallei*.

Definition of species-specific oligonucleotide primers. For the differentiation of the *B. mallei*/*B. pseudomallei* group from other *Burkholderia* species (1), sequence deviations within the region of helices 9 and 10 (Fig. 3) of the 23S rDNA were used to design sense primer VMP 23-1 specific for *Burkholderia vietnamiensis*, *B. mallei*, and *B. pseudomallei* (Table 2); those in the helix 45 region (Fig. 4) were used to design antisense primer MP 23-2 specific for *B. mallei* and *B. pseudomallei* (Table 2). A PCR with this pair of primers results in a product of 1,051 bp with template DNAs from *B. mallei* and *B. pseudomallei* but not with template DNAs from other *Burkholderia* species (Table 1).

The substitution T 2143 C within the helix 78 region of the 23S rDNA, which is unique for *B. mallei*, was used for the definition of *B. mallei*-specific antisense oligonucleotide primer M 23-2 (Table 2). In combination with sense primer CVMP 23-1 (for *Burkholderia cepacia*, *B. vietnamiensis*, *B. mallei*, *B. pseudomallei*) it allows the discrimination of *B. mallei* from the other *Burkholderia* species investigated. To enhance the specificity of the test, antisense oligonucleotide primer CVP-23-2 (Table 2), appropriate for *B. cepacia*, *B. vietnamiensis*, and *B. pseudomallei* but not for *B. mallei*, was constructed and modified at its 3′ end to block the initiation of PCR amplification (Fig. 5). With this procedure PCR products of the expected size (526 bp) were obtained for all three *B. mallei* strains investigated, while no amplification product was detectable with templates from other *Burkholderia* species (Table 1).

B. mallei and *B. pseudomallei* were assigned to rRNA homology group II according to the results of DNA-rRNA hybridization studies (13). The separation of *B. mallei* and *B. pseudomallei* into distinct species is not supported by the data from nucleic acid analysis. DNA-DNA hybridizations revealed DNA similarities of more than 80% between the two species (15), 10% above the threshold set for the separation of species (19, 23). This close relationship is confirmed by comparison of the 16S and 23S rDNA sequences. Our results as well as the results of other authors (6, 8, 20, 24) indicate complete identity of the 16S rDNA of both species. The nucleotide difference detected within the 23S rDNA at position 2143 (T in *B. mallei*, C in *B. pseudomallei*) appears to be species specific as it was present in the 23S rDNA sequences of all three *B. mallei*

TABLE 2. Oligonucleotide primers used for specific PCR

Target species	Primer[a]	23S rDNA helices containing target position	Sequence	Size of PCR product (bp)	Annealing temp (°C)
B. vietnamiensis, *B. mallei*, and *B. pseudomallei*	VMP 23-1	9ab/10a	5′-CTT TTG GGT CAT CCT RGA-3′	1,051	58
B. mallei and *B. pseudomallei*	MP 23-2	45ab/36b	5′-TCC TAC CAT GCG AGA CT-3′		
All *Burkholderia* spp.	CVMP 23-1	5b/8ab	5′-AAA CCG ACA CAG GTG G-3′		
B. mallei	M 23-2	78ab	5′-CAC CGA AAC TAG CA-3′	526	47
All *Burkholderia* spp. excluding *B. mallei*	CVP 23-2[b]	78ab	5′-CAC CGA AAC TAG CG-3′		

[a] V, *B. vietnamiensis*; M, *B. mallei*; P, *B. pseudomallei*; C, *B. cepacia*. A suffix of 1 indicates a sense primer; a suffix of 2 indicates an antisense primer.
[b] Contains an NH_2 modification at the 3′ end to supress amplification of *Burkholderia* species other than *B. mallei*.

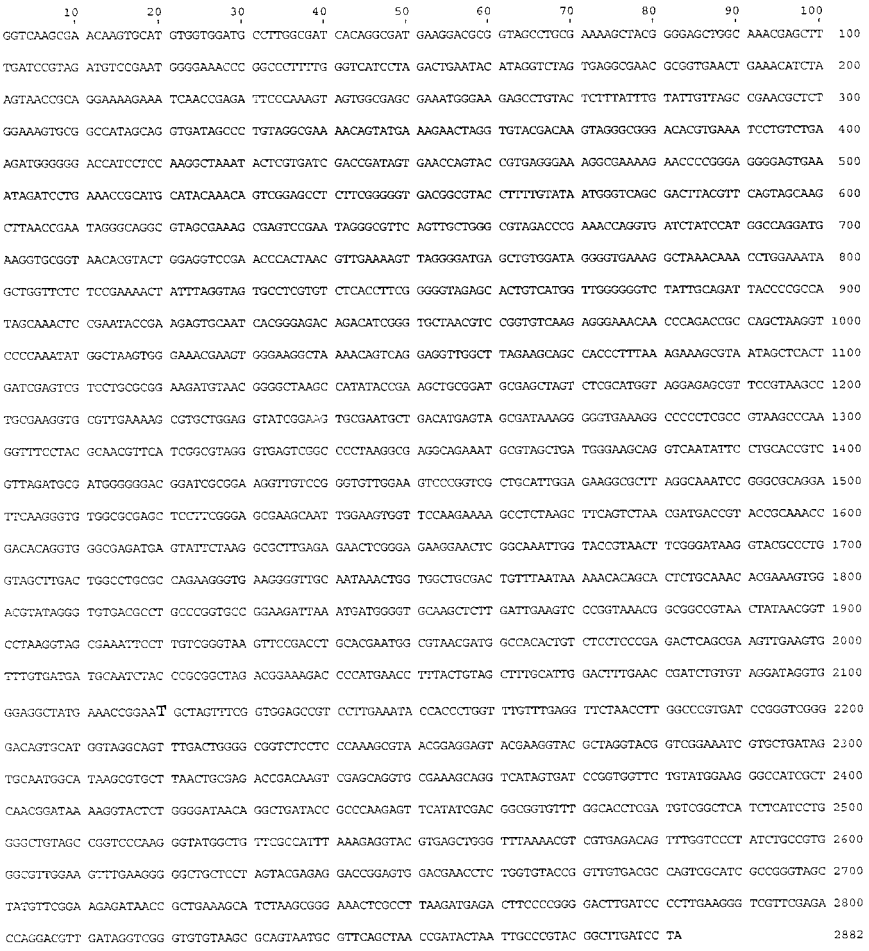

FIG. 1. 23S rDNA gene sequence of *B. mallei* ATCC 23344^T. The T at position 2120 (corresponding to 2143 in the *E. coli* numbering system described by Brosius et al. [2]), which is different from the corresponding nucleotide for all other *Burkholderia* species, is in boldface.

strains and was not detectable in any of the 15 *B. pseudomallei* strains. The nucleotide exchange is located within the more conserved domain V of the 23S rDNA (12). It therefore can be regarded as a stable species-specific character.

Another difference between species was detected by Tyler et al. (20) in the 16S-23S spacer area common for both species (G in *B. mallei* in comparison with T in *B. pseudomallei*). This signalizes a further possibility for a *B. mallei*-specific signature sequence. However, this difference appears less appropriate as it has to be regarded as less stable due to the insignificance of selective constraints within the noncoding spacer region. This difference between *B. mallei* and *B. pseudomallei* was identified by comparison of only one strain of each species. Furthermore, Kostman et al. (9) found a high level of variability within the 16S-23S spacers of different *B. cepacia* strains.

The identification of three different 23S rDNA sequences within the *B. pseudomallei* strains reveals a remarkable heterogeneity. This observation is supported by the results of DNA hybridization studies by Rogul et al. (15) indicating genetic heterogeneity of *B. pseudomallei* as well. A sequence analysis

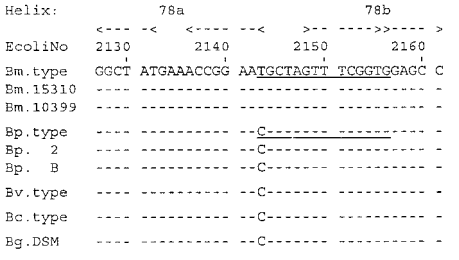

FIG. 2. Alignment of the 23S rDNA within the helix 78 region. In the sequences of all three *B. mallei* strains a T is located at position 2143 instead of C in the *B. pseudomallei* strains. Underlined nucleotides show the target signature for the *B. mallei*-specific PCR primer (M 23-2). Bm, *B. mallei*; Bp, *B. pseudomallei*; Bv, *B. vietnamiensis*; Bc, *B. cepacia*; Bg, *Burkholderia gladioli* DSM 4285.

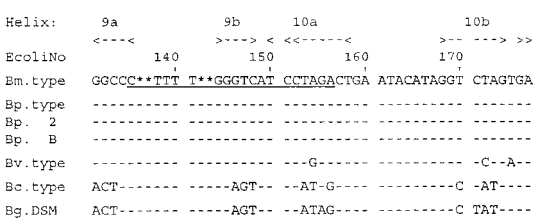

FIG. 3. Alignment of the 23S rDNA within the region of helices 9 and 10. Underlined nucleotides show the target signature specific for *B. vietnamiensis*, *B. mallei*, and *B. pseudomallei* (VMP 23-1). *, no nucleotide at this position. Species abbreviations are as defined in the legend for Fig. 2.

```
Helix:      41'b       41b            45a                45b         36b
         >------->  >---------  -->
<-----<            >- ---->     > ---->
EcoliNo       1150        1160       1170       1180        1190
Bm.type   AGCCATAT  ACCGAAGCTG  CGGATGCGAG  CT***AGTCT  CGCATGGTAG  GAGAG
Bp.type   --------  ----------  ----------  ----------  ----------  -----
Bp.  2    --------  ----------  ----------  ----------  ----------  -----
Bp.  B    --------  ----------  ----------  ----------  ----------  -----
Bv.type   ---T----  ----------  --------T-  -----TTG-A  ----------  -----
Bc.type   ---T----  ----------  --------T-  -----TTG-A  ----------  -----
Bg.DSM    ---T----  ----------  -------ATA  *----TTATA  T---------  -----
```

FIG. 4. Alignment of the 23S rDNA within the helix 45 region. Underlined nucleotides show the target signature specific for *B. mallei* and *B. pseudomallei* (MP 23-2). Species abbreviations are as defined in the legend for Fig. 2.

of the 16S-23S intercistronic spacers (20) demonstrated heterogeneity within the same strain (*B. pseudomallei* type strain). This heterogeneity may be useful for genotyping *B. pseudomallei* strains from different origins. It appears worthwhile to elucidate the degree of relationship between different lines of descent within the species *B. pseudomallei*.

The two-species concept for *B. mallei* and *B. pseudomallei* is based on major differences between them in their phenotypes (e.g., biochemical activities) and in the clinical symptoms and epidemiologies of the diseases they cause. These differences justify the definition of *B. mallei* and *B. pseudomallei* as two distinct species in the modern understanding of taxonomy, which is polyphasic (4, 21), integrating phenotypic, genotypic, and phylogenetic information.

In medical microbiology unequivocal identification of *B. mallei* and *B. pseudomallei* by conventional biochemical reactions is usually achieved. There is, however, a remarkably high risk of becoming infected while working with living cultures of *B. mallei* or *B. pseudomallei*. This risk could be significantly reduced by using the identification procedure described. The speciation part of the laboratory work can then be performed with killed bacteria or the template DNA thereof. Apart from the reduction of the risk of infection, the time necessary for speciation can be reduced to about 3 to 4 h in comparison with 2 days for conventional identification. Furthermore, the procedure can be adapted for use for in situ hybridization in clinical specimens (22).

Nucleotide sequence accession numbers. The complete 23S rDNA gene sequences of the cited *B. pseudomallei* and *B. mallei* strains will appear in the EMBL Database under the accession no. Y17183 (*B. mallei* ATCC 23344^T) and Y17184 (*B. pseudomallei* ATCC 23343^T).

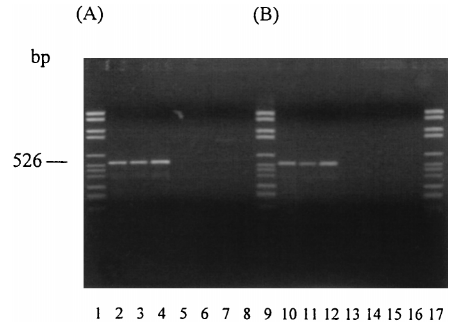

FIG. 5. PCR with a *B. mallei*-specific primer combination. (A) PCR without a competitive *B. pseudomallei*-directed 3′-modified probe (primers CVMP 23-1 and M 23-2); (B) PCR with a competitive *B. pseudomallei*-directed 3′-modified probe (primers CVMP 23-1, M 23-2, and CVP-23-2 [3′-modified]). Lanes 1, 9, and 17, DNA molecular weight marker; lanes 2 and 10, *B. mallei* ATCC 23344^T; lanes 3 and 11, *B. mallei* ATCC 15310; lanes 4 and 12, *B. mallei* ATCC 10399; lanes 5 and 13, *B. pseudomallei* ATCC 23343^T; lanes 6 and 14, *B. pseudomallei* ATCC 15682; lanes 7 and 15, clinical isolate of *B. pseudomallei*; lanes 8 and 16, negative controls without template DNA. No difference between the lanes with or without competitive primers is detectable.

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